

AUGMENTATION OF MACROPHAGE COMPLEMENT RECEPTOR FUNCTION IN VITRO

I. Characterization of the Cellular Interactions Required for the Generation of a T-Lymphocyte Product that Enhances Macrophage Complement Receptor Function*

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The plasma membranes of phagocytic leukocytes have receptors for the Fc portion of IgG (1-6) and for a cleavage product of the third component of complement, C3 (3, 4, 6-8). These receptors are distinct from one another (1-3) and serve different functions in promoting the interaction of phagocytic cells with immunologically coated particles and soluble material in the cells' environment (6-10). Several investigators have demonstrated that the C3b receptors of mouse peritoneal macrophages (6-9) and other phagocytic cells (11) mediate binding, but not ingestion, of C3b-coated particles. Bianco et al. (8) have previously found that the C3b receptors of mouse peritoneal macrophages elicited by the i.p. injection of thioglycollate medium mediate both binding and ingestion of C3b-coated particles. It was not possible in those studies to determine whether thioglycollate medium altered macrophage complement receptor function by inducing the influx of a physiologically different population of mononuclear phagocytes into the peritoneal cavity, by altering the number or density of complement receptors of resident peritoneal macrophages, or by inducing a qualitative change in the function of complement receptors of the resident macrophage population.

To understand better the cellular and molecular mechanisms by which such changes in complement receptor function may occur in vivo, we undertook to determine the in vitro conditions under which the function of macrophage complement receptors could be modified. Because available evidence suggests that thioglycollate-elicited macrophages behave similarly in many respects to macrophages that have been influenced by lymphokines (12-15), our initial efforts were directed at examining the effects of antigen-stimulated lymphocytes and their products upon macrophage complement receptor function. However, in experiments in which lymphocytes and macrophages from bacille Calmette Guérin-infected mice were cultivated together in the presence of appropriate antigen, we were unable to detect any

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alteration of macrophage complement receptor function (J. A. Griffin and F. M. Griffin, unpublished observations).

A variety of soluble factors that influence macrophage behavior has been described previously (16). For the most part, these products are elaborated by T lymphocytes and alter aspects of macrophage function as diverse as motility (17–20), glucose and oxygen uptake and metabolism (14, 15, 21), cytotoxicity (22, 23), phagocytic activity (14, 15, 24), microbicidal and microbistatic capability (25–27), protease secretion (28–30), and the interactions of macrophages with other lymphocyte populations (31). The requirements for the generation of these T-lymphocyte products are generally unique to the specific product and to the macrophage function affected. Some lymphocyte products are elaborated only after a signal is received by lymphocytes from macrophages or macrophage products (31–33); in one experimental system, only macrophages that had recently engaged in phagocytosis were able to impart the necessary signal (31, 32).

In this paper, we report the results of experiments in which macrophage complement receptor function may be converted in vitro from mediating only particle binding to promoting both binding and ingestion of complement-coated particles. Our results show that a T-lymphocyte product is responsible for the observed change in macrophage complement receptor function. To elaborate the product, lymphocytes must receive a signal, not from an antigen, but from macrophages that have been triggered by phagocytosis of IgG-containing immune complexes. The findings presented may have physiologic relevance, because the in vitro conditions required to effect enhanced complement receptor activity are similar to the conditions thought to prevail at sites of inflammation in vivo.

Materials and Methods

Reagents and Media. Glutaraldehyde, 50% aqueous solution (Fisher Scientific Co., Pittsburgh, Pa.); sheep erythrocytes (E)¹ (Animal Blood Center, Syracuse, N.Y.); polystyrene latex beads, 1.1 μ in Diam (Dow Corning Corp., Midland, Mich.); fetal bovine serum (FBS) and freshly frozen guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.); Medium 199 (medium) (Microbiological Associates, Walkersville, Md.); and Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) were obtained from the manufacturers as indicated. FBS was decplemented by heating at 56°C for 30 min before use. Brewer thioglycollate medium was obtained from Difco Laboratories, Detroit, Mich.; a 4.05% aqueous solution was prepared according to the manufacturer's directions. Lyophilized rabbit gamma globulin (N. L. Cappel Laboratories Inc., Cochranville, Pa.) was reconstituted in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml; a portion of the reconstituted material was aggregated at 63°C for 45 min (34). Lyophilized human serum albumin (HSA, recrystallized five times, Sigma Chemical Co., St. Louis, Mo.) was reconstituted in PBS at a concentration of 20 mg/ml.

Antibody Preparations. Rabbit anti-sheep E IgG and rabbit anti-sheep E IgM were obtained from Cordis Laboratories Inc., Miami, Fla. Rabbit antiserum against the capsular polysaccharide of type 1 *Streptococcus pneumoniae* was obtained from the New York State Department of Health, Albany, N. Y. Rabbit anti-ox E IgG was a gift from Dr. E. Pearl, University of

¹ *Abbreviations used in this paper:* AI, attachment index; E, erythrocytes; E(IgG), E coated with anti-E IgG; E(IgG)C, E(IgG) coated with the first four complement components; E(IgM), E coated with anti-E IgM; E(IgM)C, E(IgM) coated with the first four complement components; E(IgM)C3d, E(IgM)C incubated for 1 h with a source of C3b inactivator; FBS, fetal bovine serum; HSA, human serum albumin; medium, Medium 199; oxE(IgG), 0.5% ox E coated with anti-ox E IgG; PBS, phosphate-buffered saline; PI, phagocytic index.

Alabama in Birmingham, Birmingham, Ala. Fluorescein-tagged goat anti-mouse μ -chain antibody was a gift from Dr. A. Lawton, University of Alabama in Birmingham. It was prepared by immunizing a goat with purified mouse myeloma IgM; anti-mouse μ -chain antibodies were purified by immunoselection of the antiserum on Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) (35). Rhodamine-tagged rabbit anti-rat thymocyte IgG was a gift from Dr. C. Balch, University of Alabama in Birmingham. It was prepared by immunizing a rabbit with rat thymocytes and selecting the IgG fraction of the antiserum (36). The specificity of the resulting preparation for mouse thymocytes and T lymphocytes has been previously documented (37). Rabbit anti-mouse Thy-1 antiserum, kindly provided by Dr. R. Zwerner, University of Alabama in Birmingham, was raised in a rabbit against purified Thy-1 preparations from the T-cell lymphoma BW5147. Its specificity for mouse thymocytes and T lymphocytes has been previously demonstrated (38). Rabbit anti-HSA antiserum was a gift from S. Jackson, University of Alabama in Birmingham.; it was heat decomplemented (56°C, 30 min) before use.

Preparation of Antibody- and Complement-coated Sheep E. E, E coated with anti-E IgM (E[IgM]), E(IgM) coated with the first four complement components (E[IgM]C)², E(IgM) incubated for 1 h with a source of C3d inactivator (E[IgM]C3d), E coated with anti-E IgG (E[IgG]), and E(IgG) coated with the first four complement components (E[IgG]C) were prepared as previously described (7, 8).

Preparation of Antibody-coated Ox E. Anti-ox E IgG was found to have an opsonic titer for sheep E at a dilution of 1:256. It was therefore absorbed with a 50% (vol/vol) suspension of sheep E at 37°C for 1 h, after which it was unable to promote either attachment or ingestion of E or ingestion of E(IgM)C by normal macrophages. Because very low concentrations of IgG have been shown to promote the ingestion of particles coated with C3b (9), our finding that the absorbed antibody preparation was unable to induce the ingestion of E(IgM)C by normal macrophages indicates that anti-sheep E antibodies had been thoroughly removed by the absorption procedure. The preparation retained full opsonic activity for ox E.

1 ml of 2% (vol/vol) ox E in medium was incubated with an equal volume of a 1:128 dilution of absorbed anti-ox E IgG for 15 min at 37°C. The suspension was centrifuged for 5 min at 750 g, and the pelleted E were suspended in medium at a concentration of 0.5%. This preparation, 0.5% ox E coated with anti-ox E IgG, is designated oxE(IgG).

Preparation of Soluble Immune Complexes. 1 ml of anti-HSA antiserum was incubated with 10 mg of HSA for 1 h at 37°C. Although the exact antigen-antibody ratio cannot be determined, the mixture contained an excess of antigen, the estimated molar ratio being at least 10:1, assuming 25% of the IgG in the antiserum was specific for HSA. No precipitates of a size sufficient to cloud the solution were formed.

Animals. 20- to 30-g female mice (strain CD-1) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.; female BALB/c mice and congenitally athymic (nu/nu) BALB/c mice were obtained from Dr. J. McGhee, University of Alabama in Birmingham.; and female C57BL/6 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. These strains served as sources of peritoneal macrophages and of splenic, mesenteric lymph node, and thymic lymphocytes. Female C5-deficient AKR mice were obtained from The Jackson Laboratory and were used as sources of C5-deficient mouse serum, as previously described (7).

Macrophages. Mouse peritoneal macrophages were harvested by peritoneal lavage and cultivated in 35-mm Petri dishes with or without 13-mm Diam glass cover slips in medium alone or medium containing 20% FBS, as previously described (7). Thioglycollate-elicited macrophages were harvested 4 d after i.p. injection of 1 ml/mouse of thioglycollate medium and cultivated under conditions identical to those described for nonelicited macrophages.

Lymphocytes. Lymphocytes were harvested from mouse spleens, mesenteric lymph nodes, and thymi as previously described (39). More than 98% of the harvested cells were lymphocytes, as determined by morphology and their inability to phagocytize polystyrene latex beads; >95% of the cells were viable, as judged by trypan blue dye exclusion.

Populations of lymphocytes enriched in B lymphocytes were prepared by two methods: (a)

² The presumed form of C3 on E(IgM)C and E(IgG)C is C3b.

splenic lymphocytes were incubated in medium at a concentration of 2×10^7 per ml at 4°C for 30 min with a 1:20 dilution of anti-Thy-1 antiserum. Cells were washed, resuspended in medium containing 5% fresh guinea pig serum as a complement source, and incubated for 15 min at 37°C . Cold medium was added and the cells were suspended in Tris buffer, layered onto Ficoll-Hypaque, and centrifuged at 400 *g* for 40 min at 25°C to remove dead cells and cellular debris. The cells in the buffer-Ficoll-Hypaque interface were washed and resuspended in medium. (b) Splenic lymphocytes from congenitally athymic (nu/nu) BALB/c mice served as a second source of B-enriched lymphocytes.

Populations of lymphocytes enriched in T lymphocytes were prepared in the following manner. Splenic lymphocytes (1×10^6 per ml) were incubated with 1.0% E(IgG)C for 15 min at 37°C . 3 ml of each lymphocyte-E suspension was layered onto a 3-ml cushion of Ficoll-Hypaque in 15-mm-Diam glass tubes and centrifuged at 400 *g* for 40 min at 25°C . The cellular pellicles at the medium-Ficoll-Hypaque interface were gently aspirated, washed in medium, and resuspended in medium or in medium-FBS at a concentration of 1×10^7 cells per ml. The interface cells contained <3% lymphocytes which, on retesting, bound three or more E(IgG)C.

Bacteria. *S. pneumoniae* type 1 was kindly provided by Dr. H. Dillon, University of Alabama in Birmingham. The bacteria were maintained, passed in mice, and cultivated as previously described (7). Some pneumococci were opsonized by incubating $1-2 \times 10^8$ organisms with heat-inactivated (56°C , 30 min) anti-capsular antiserum (180 μg protein) in 1 ml of medium at 4°C for 30 min (7). Two strains of *Escherichia coli*, originally isolated from the blood of patients, were maintained on blood agar plates and grown overnight in brain heart infusion broth. Before use, they were washed twice and resuspended in medium. $1-2 \times 10^8$ bacteria were added to each macrophage monolayer culture.

Fluorescence Microscopy. Some lymphocyte cultures were cytocentrifuged onto glass cover slips and incubated at 4°C sequentially with fluorescein-tagged goat anti-mouse μ -chain antibody and rhodamine-tagged rabbit anti-rat thymocyte IgG. These preparations were examined with a Leitz Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) equipped with a C5 100 W/2 mercury vapor lamp (Philips Electronic Instruments, Inc., Mahwah, N. J.), a Ploem epi-illumination system, dichroic mirrors, and filters selective for fluorescein and rhodamine (40).

Generation of Experimental Supernates. 2×10^6 nonelicited (resident) peritoneal cells ($\approx 1 \times 10^6$ macrophages) or 1.25×10^6 thioglycollate-elicited peritoneal exudate cells ($\approx 1 \times 10^6$ macrophages) were plated in culture dishes and incubated for 1 h at 37°C , after which dishes were washed twice with medium to remove nonadherent cells. The adherent cell population (effector macrophages³) was overlaid with fresh medium without FBS; 0.2 ml of 0.5% E(IgG) was added to each macrophage monolayer and incubation continued for 4 h at 37°C . Cultures were then washed and noningested E lysed with NH_4Cl . 5×10^6 splenic or mesenteric lymph node lymphocytes in either medium alone or medium-FBS⁴ were added to each dish and incubation continued for 24 h at 37°C . Supernatant medium from each culture was harvested, centrifuged at 750 *g* for 10 min to remove cells and cellular debris, and assayed for its effect upon macrophage complement receptor function, as described below. Only variations from these standard procedures are described in the text.

Assessment of Supernatant Activity. Cover slip cultures of freshly explanted, nonelicited mouse peritoneal macrophages were incubated in either experimental supernatant media, medium alone, or medium-20% FBS for 48 h at 37°C , after which supernates and media were removed and cultures washed twice with fresh medium. The complement receptor activity of these assay macrophages was determined by incubating the monolayers with 0.2 ml of 0.5% E(IgM)C in fresh medium for 30 min at 37°C . Cultures were then washed and fixed with glutaraldehyde;

³ Macrophages that were used in the generation of supernates will be referred to as effector macrophages; those used to assess the activity of supernates will be referred to as assay macrophages. All experiments were performed using both thioglycollate-elicited and nonelicited adherent peritoneal cells as effector macrophages. Because the physiologic state of the effector macrophages did not substantially influence the activity of supernates, results are presented without regard to the source of these cells.

⁴ All experiments were performed using medium alone and using medium-20% FBS. There were no substantial differences between supernates prepared in medium-FBS and those prepared in medium alone.

E attachment and ingestion were scored by phase contrast microscopic examination (7).

Presentation of Results. Each result given represents the average of at least three separate determinations, each performed in duplicate. Attachment and phagocytic indices (AI and PI) are the number of E attached (AI) or ingested (PI) by 100 macrophages and were obtained by multiplying the percentage of macrophages that had attached or ingested any E by the average number of E attached or ingested per macrophage. Some results are presented as the percentage of the PI obtained under standard conditions of supernate generation. In the Figures, each result is presented as mean \pm SEM.

Results

Ingestion of E(IgM)C by Supernate-treated Macrophages. Our initial observation was that macrophages treated with supernates prepared as described in Materials and Methods acquired the ability to ingest E(IgM)C. 59% of these macrophages ingested an average of 6.0 E(IgM)C each, for a PI of 354, whereas their nontreated counterparts ingested very few E(IgM)C (Table I). That ingestion of these E was due to enhanced C3b receptor function and not to the acquisition by supernate-treated macrophages of receptors for either E themselves or another immunologic component on the E surface is also demonstrated by the results presented in Table I. Like their nontreated counterparts, supernate-treated macrophages did not bind or ingest either E, E(IgM), or E(IgM)C3d.

Cellular Requirements for the Generation of Supernatant Activity. Supernates were prepared from cultures containing either macrophages, lymphocytes, or E(IgG) alone; from cultures containing only two of the three cell types; from cultures containing macrophages, lymphocytes, and anti-E IgG without E; and from cultures containing macrophages, lymphocytes, and noncoated E. As shown in Table II, none of these supernatant preparations produced a significant change in the assay macrophages' interaction with C3b-coated E. Thus, the generation of supernatant activity required interactions between effector macrophages and E(IgG) and subsequently between these macrophages and lymphocytes.

Effects of Low Temperature and of Nonviable Cells upon the Generation and Expression of Supernatant Activity. Supernates prepared from cultures containing either heat-killed macrophages (which had previously ingested E(IgG)) or heat-killed lymphocytes were inactive, as were supernates prepared from cultures in which lymphocyte-macrophage cocultivation was performed at 4°C (data not shown). In addition, assay macrophages that were incubated with active supernates at 4°C failed to ingest E(IgM)C (data not shown). These results suggest that both the generation and expression of supernatant activity require cellular metabolism.

Effects of Varying Cell Numbers upon the Activity of Supernates. Fig. 1a records the results obtained when the number of effector macrophages employed was varied from 0 to 1×10^6 per culture. In Fig. 1b, the number of lymphocytes employed was varied from 0 to 5×10^6 per culture. Fig. 1c records the results obtained when the concentration of E(IgG) added in 0.2-ml aliquots to each culture was varied from 0 to 0.5%. The activity of supernates generated was directly dependent upon the concentration of each of the cell types used.

Effects of Varying Incubation Times upon the Activity of Supernates. As depicted in Fig. 2a, the potency of supernates was directly proportional to the duration of incubation of effector macrophages with E(IgG). Examination of parallel cultures of effector macrophages cultivated on cover slips indicated that the number of E(IgG) ingested

TABLE I
Interaction of Assay Macrophages with Various Preparations of Sheep E

Particle added	Nontreated macrophages		Supernate-treated macrophages	
	AI*	PI*	AI	PI
E	12	0	9	2
E(IgM)	14	2	17	4
E(IgM)C3d	15	3	14	7
E(IgM)C3b	≈ 1,200	4	≈ 1,300	354

* Calculated as described in Materials and Methods.

TABLE II
Cellular Requirements for the Generation of Supernatant Activity

Cells present in supernate-generation steps			PI of E(IgM)C by assay macrophages
E(IgG)	Effector macrophages	Lymphocytes	
+	+	+	397
+	+	—	15
+	—	—	12
+	—	+	23
—	+	+	16
—	+	—	15
—	—	+	10
—	—	—	13
Anti-E IgG*	+	+	32
E only‡	+	+	8

* Anti-E IgG only (without E) was used.

‡ Uncoated E were used.

per macrophage increased with the duration of incubation (data not shown). These results suggest that the potency of supernatant activity is directly related to the number of E(IgG) ingested by effector macrophages.

The duration of lymphocyte-macrophage cocultivation was also varied, from 0 to 24 h (Fig. 2b). Maximally potent supernatant activity was generated after 10 h of lymphocyte-macrophage cocultivation.

In other experiments, the duration of incubation of assay macrophages with supernatant media was varied from 0 to 48 h. Assessment of the macrophages' ability to ingest E(IgM)C was always performed after 48 h of macrophage in vitro cultivation, and supernates were always present at the 48th h of incubation. For example, macrophages which were exposed to supernates for 4 h were incubated in medium or medium-FBS from the time of explanation until the 44th h of in vitro cultivation and in active supernates for the last 4 h. As demonstrated in Fig. 2c, the effect of supernates upon macrophage complement receptor function was detected after 6 h of incubation, but maximal effect was not achieved until after 48 h of incubation of macrophages with supernates.

Effect of Varying Concentrations of Supernates. Varying dilutions of maximally effective supernates were prepared in medium or medium-FBS and their effect upon macrophage complement receptor function assayed. As demonstrated in Fig. 3, supernates augmented macrophage complement receptor function in a dose-dependent manner.

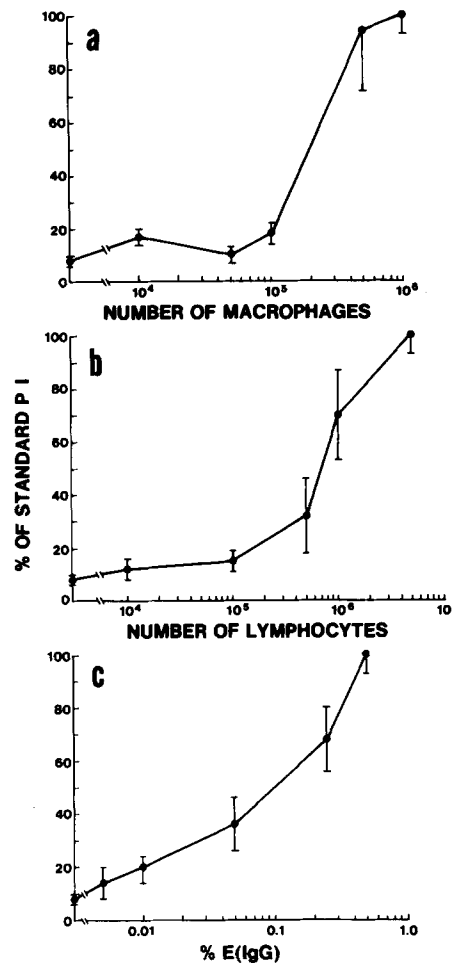


FIG. 1. Effect of varying cell numbers upon the activity of supernatant media generated. (a) The number of effector macrophages was varied from 0 to 1×10^6 per culture. (b) The number of lymphocytes was varied from 0 to 5×10^6 per culture. (c) The percentage of E(IgG), added in 0.2-ml aliquots per culture, was varied from 0 to 0.5%. The percentage of standard PI (ordinate) is the percentage of the PI obtained when the numbers of cells used to generate supernates were as described in Materials and Methods.

Duration of Enhanced Macrophage Complement Receptor Function after Removal of Supernates. Assay macrophages that had been incubated for 48 h at 37°C in active supernates were washed, overlaid with fresh medium, and incubated at 37°C. At varying times thereafter, the macrophages' complement receptor function was assessed. As shown in Fig. 4, the effect of supernates was quite transient; the ability of macrophages to ingest E(IgM)C was reduced by 50% within 30 min and was virtually abolished within 4 h of removal of supernates.

Effect of Phagocytosis of Various Particles upon the Macrophages' Ability to Participate in the Generation of Active Supernates. The next group of experiments was designed to determine whether phagocytosis of any particle by effector macrophages was sufficient to trigger the macrophage function necessary for the generation of active supernates or whether Fc receptor-mediated phagocytosis was required.

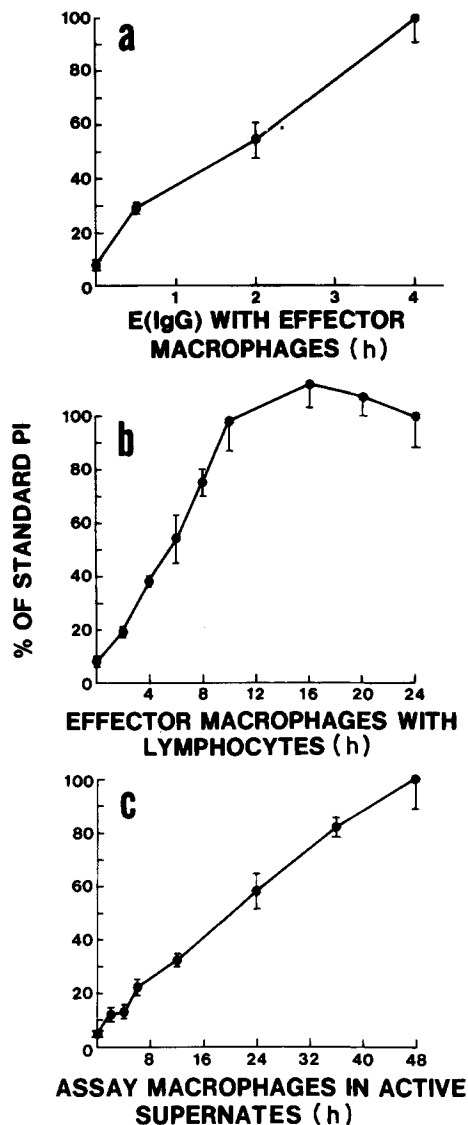


FIG. 2. Effect upon the generation and expression of supernatant activity of varying the duration of incubation of: (a) E(IgG) with effector macrophages, (b) effector macrophages with lymphocytes, and (c) assay macrophages in active supernatant media. The percentage of Standard PI (ordinate) is the percentage of the PI obtained when the incubation conditions were as described in Materials and Methods.

Effector macrophages were incubated for 4 h at 37°C with various particles and their ingestion determined by phase contrast microscopic examination (Table III, column 2). Group I included polystyrene latex beads, *E. coli*, and zymosan: particles that are ingested by means not known to involve the participation of specific phagocytic receptors. Group II included E(IgM)C and complement-coated zymosan: particles that interact with macrophages via the macrophages' C3 receptors; these particles were avidly bound by nonelicited effector macrophages (data not shown)

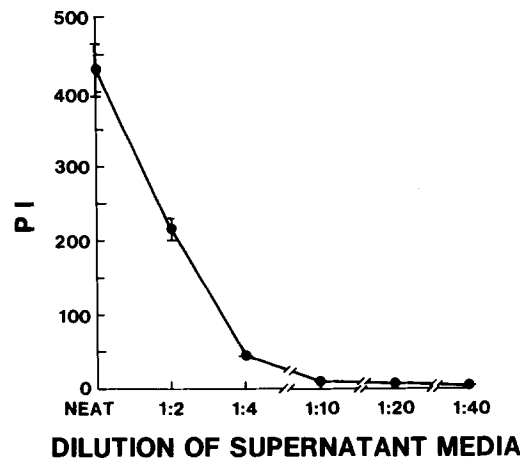


FIG. 3. Dose response of the effect of supernatant media upon macrophage complement receptor function. Supernates were prepared as described in Materials and Methods. The term neat refers to the entire volume of supernatant medium in one culture dish; other notations on the abscissa are dilutions of the neat supernate with medium-20% FBS. PI (ordinate) is the number of E(IgM)C ingested by 100 assay macrophages.

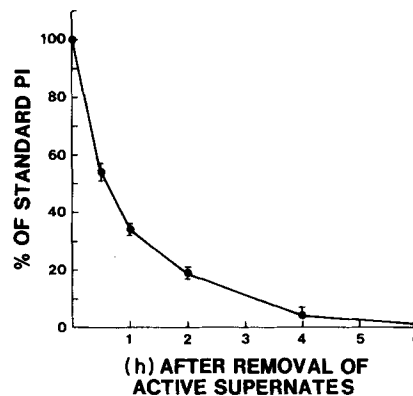


FIG. 4. Duration of augmented macrophage complement receptor function after removal of supernates. Assay macrophages were incubated in active supernates for 48 h; supernates were removed and macrophages were washed, covered with fresh medium, and placed at 37°C. At varying times thereafter, macrophage complement receptor function was assessed. The percentage of Standard PI (ordinate) is the percentage of the PI of macrophages treated with supernates generated as described in Materials and Methods.

and were readily ingested by thioglycollate-elicited effector macrophages. Group III included sheep E(IgG), oxE(IgG), and IgG-coated *S. pneumoniae*: particles whose ingestion is mediated by the macrophages' Fc receptors.

The supernatant media generated from cultures containing lymphocytes and these macrophages were assessed for their effect upon the complement receptor function of assay macrophages; the results are presented in Table III, column 3. Supernates generated from cultures containing macrophages that had phagocytized via nonimmunologic means (lines 1-3) or that had either bound or ingested particles via their complement receptors (lines 4 and 5) were without effect upon the complement

TABLE III
Effect of Incubating Effector Macrophages with Various Particles upon the Macrophages' Ability to Participate in the Generation of Supernatant Activity

Group	Particle incubated with effector macrophages	PI of test particle by effector macrophages	PI of E(IgM)C by assay macrophages
I	<i>E. coli</i>	1,400	11
	Latex beads	1,400	12
	Zymosan	750	22
II	E(IgM)C	1,200	8
	Complement-coated zymosan	900	18
III	E(IgG)	1,300	360
	oxE(IgG)	1,260	351
	IgG-coated <i>S. pneumoniae</i>	1,400	158

receptor function of assay macrophages. All supernates generated from cultures containing macrophages that had phagocytized IgG-coated particles (lines 6–8), on the other hand, exerted a striking effect upon macrophage complement receptor function.

These results demonstrate that the stimulus for the effector macrophages' participation in the generation of active supernates is not phagocytosis per se, but that phagocytosis via the cells' Fc receptors is both the necessary and sufficient trigger for these macrophages to interact with lymphocytes to produce a factor(s) that enhances the complement receptor function of assay macrophages.

Soluble Immune Complexes Can Substitute for IgG-coated Particles in Triggering the Macrophage Function Required for the Generation of Supernatant Activity. Two types of soluble IgG-containing material, heat-aggregated IgG, which has been previously found to mimic soluble immune complexes in many respects (34, 41), and HSA-anti-HSA complexes, were used in place of IgG-coated particles to engage Fc receptors of effector macrophages. Control macrophage cultures received either nonaggregated IgG, anti-HSA antiserum alone, or HSA plus nonimmune rabbit serum. Supernates from cultures containing macrophages that had been fed either aggregated IgG or HSA-anti-HSA complexes were as active as those prepared using IgG-coated particles, whereas all control supernates were inactive (Fig. 5). Thus, either soluble or particulate IgG-containing immune complexes can trigger effector macrophages to perform their function in the generation of supernatant activity.

The Activity of Supernates Is Dependent upon the Number of Effector Macrophage Fc Receptors Engaged by IgG Molecules. The following experiments were designed to determine whether the quantity of IgG contained in ingested immune complexes, or the number of IgG-coated particles phagocytized by effector macrophages, regulated the activity of supernates. Here we took advantage of the finding of Ehlenberger and Nussenzweig (9) that the number of IgG molecules required to trigger the ingestion of a particle coated with both IgG and C3b molecules is much less than the number required for ingestion of a particle coated with only IgG. E(IgM)C were incubated with varying quantities of anti-E IgG, the greatest quantity used (5 μ l/ml) being the amount normally used to prepare E(IgG). These complement- and IgG-coated E were then

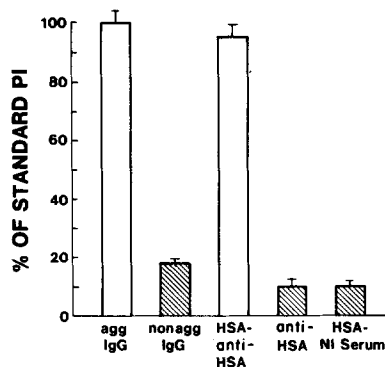


FIG. 5. Effect of ingestion of aggregated IgG and of soluble immune complexes by effector macrophages upon the activity of supernates. Either 2 mg of aggregated rabbit (agg) IgG, soluble HSA-anti-HSA complexes containing 2 mg of HSA, or a control preparation was substituted for E(IgG) as the material fed to effector macrophages. Other conditions of supernate generation and assay were as described in Materials and Methods. The percentage of Standard PI (ordinate) is the percentage of the PI of macrophages treated with supernates generated as described in Materials and Methods. NI serum, nonimmune rabbit serum; nonagg IgG, nonaggregated rabbit IgG.

incubated with effector macrophages (both thioglycollate-elicited and nonelicited) for 4 h at 37°C. Thioglycollate-elicited macrophages ingested all E preparations equally well, ingestion being mediated via both Fc and C3b receptors presumably. Nonelicited macrophages ingested equally well E prepared with 5, 2.5, and 1 μ l/ml of anti-E IgG, but ingested E prepared with 0.5 and 0.1 μ l/ml much less avidly (Fig. 6a).

These macrophages, which had ingested E coated with varying quantities of anti-E IgG, were cocultivated with lymphocytes for 24 h at 37°C to prepare experimental supernates. The activity of supernates was directly dependent upon the quantity of anti-E IgG on the E surface and independent of the number of IgG-coated E ingested (Fig. 6b). Thus, the number of effector macrophage Fc receptors engaged by particle-bound IgG regulates the activity of supernatant media. The number of IgG-coated particles ingested appears to be important only insofar as it reflects the number of Fc-Fc receptor interactions.

Identification of the Lymphocytes that Participate in the Generation of Supernatant Activity. When either thymocytes or T-lymphocyte-enriched spleen cell preparations were used in place of nonfractionated splenic lymphocytes, the resulting supernates were active (Table IV). However, supernates prepared from cultures in which either B lymphocyte-enriched spleen cells or splenic lymphocytes from athymic (nu/nu) mice were used were inactive (Table IV). These results indicate that T lymphocytes perform the necessary and sufficient lymphocyte function in the generation of active supernates.

Identification of the Cell Type that Elaborates the Active Principle. Effector macrophages that had ingested E(IgG) were incubated with thymocytes at 37°C for 12 h, at which time thymocytes were aspirated, washed, adjusted to 5×10^6 cells per culture in medium or medium-FBS, and incubated alone for 12 h at 37°C. The effector macrophages from these cultures were washed, treated with anti-Thy-1 antiserum and complement to remove remaining thymocytes, covered with fresh medium or medium-FBS, and incubated for 12 h at 37°C. Examination of parallel cultures revealed that treatment with anti-Thy-1 antiserum and complement removed contaminating thy-

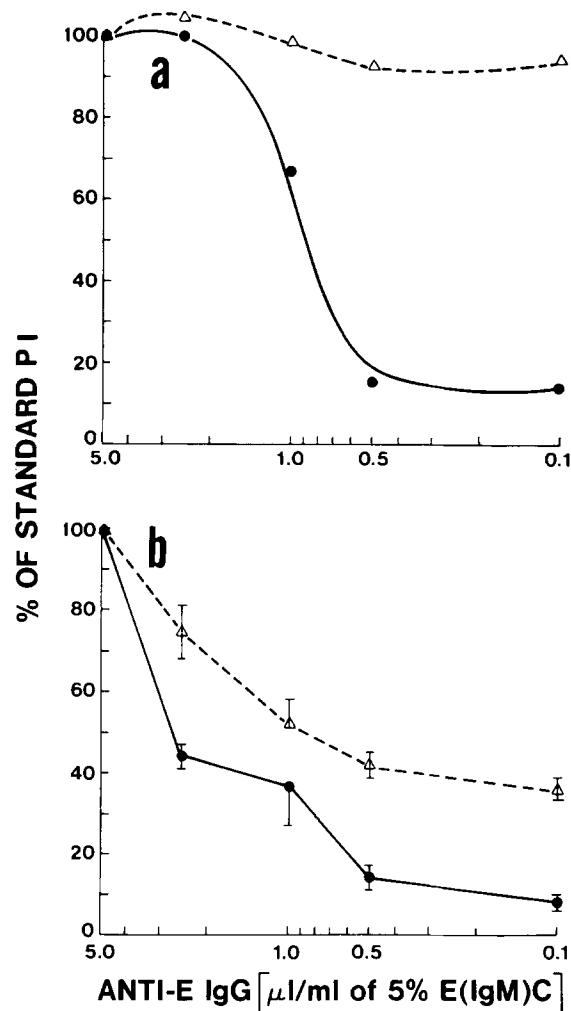


FIG. 6. Effect of the quantity of IgG on the particle ingested by effector macrophages upon the activity of supernates. E(IgM)C were prepared as described in Materials and Methods. These E(IgM)C were then incubated with varying quantities of anti-E IgG. The resulting E(IgM)C-(IgG) were incubated for 4 h with either nonelicited (●) or thioglycollate-elicited (Δ) effector macrophages. Ingestion of these E by effector macrophages is recorded in (a). Other conditions of supernate generation were as described in Materials and Methods. Supernatant medium from each of these cultures was then assessed for its effect upon the complement receptor function of normal macrophages (b).

mocytes and that macrophages in these cultures remained morphologically intact, excluded trypan blue dye, and were able to phagocytize normally. The thymocyte and macrophage supernates were then tested separately for their ability to augment macrophage complement receptor function. Thymocyte supernates were $92 \pm 10\%$ as active as those prepared in the standard fashion. Supernates from macrophage cultures, on the other hand, were completely inactive. Therefore, T lymphocytes, but not macrophages, elaborate the active principle.

Requirement for Macrophage-T Lymphocyte Contact in the Generation of Active Supernates. Macrophages and lymphocytes have been reported to interact in both contact-

TABLE IV
Effect of Lymphocyte Subpopulations upon the Generation of Supernatant Activity

Source	Lymphocytes		Percentage of		Percentage of standard PI
	Treatment		B cells	T cells	
			%		%
Spleen	—		43	53	100
Spleen	E(IgG)C rosetted (Ficoll-Hypaque pellicle)		10	66	95
Thymus	—		2	89	90
Spleen	Anti-Thy-1-antiserum + complement		97	3	12
Athymic (nu/nu) mouse spleen	—		90	3	8

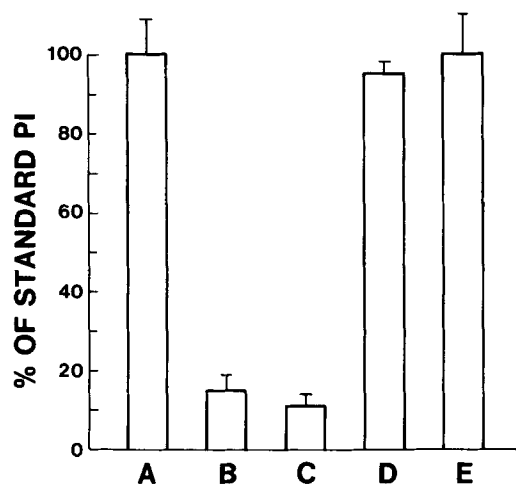


FIG. 7. Effect of lymphocyte-macrophage contact upon the generation of supernatant activity. Experimental supernates were generated as described in Materials and Methods (bar A). Fc receptor-triggered macrophages were cultivated for 12 h at 37°C, after which thymocytes were incubated for 12 h at 37°C in the macrophage culture supernates (bar B). Thymocytes and Fc receptor-triggered effector macrophages were separated by (C) or were overlaid with (D) a Millipore filter (0.22- μ m pore size). In (E), supernates prepared as described in Materials and Methods were preincubated with Millipore filters for 2 h at 37°C before transfer to assay macrophages. The activity of each supernate was assessed as described in Materials and Methods.

dependent and contact-independent manners to modulate the functions of either or both cell types (14-33, 39, 42-57). To understand better the mode of interaction between these two cell types in the generation of active supernates, we performed the following experiments.

Effector macrophages that had ingested oxE(IgG) were incubated alone for 12 h at 37°C, and the supernatant media harvested. Freshly harvested thymocytes (5×10^6 per culture) were incubated in these supernates for 12 h at 37°C, after which the culture supernates were tested for their ability to augment macrophage complement receptor function. As shown in Fig. 7, bar B, these supernates were inactive. These results suggest that effector macrophages may signal T lymphocytes to elaborate the active principle by a contact-dependent mechanism rather than by means of a soluble

macrophage mediator. However, because it was possible that, in these experiments, a soluble macrophage mediator may have been generated but subsequently degraded during the initial 12-h incubation, the necessity for lymphocyte-macrophage contact was further examined in the following experiments.

Effector macrophages were fed oxE(IgG). Each macrophage monolayer was then gently covered with a Millipore filter (Millipore Corp., Bedford, Mass.) of 0.22- μ m pore size, 5×10^6 thymocytes were added to each culture dish, and incubation continued for 24 h at 37°C. To be certain that the Millipore filters had not crushed or otherwise injured the macrophage monolayers, parallel cultures were examined by phase contrast microscopy after incubation with trypan blue dye. The morphology of the macrophages was normal, and >98% of the cells excluded the dye. The supernatant media were aspirated, freed of thymocytes and cellular debris by centrifugation, and assayed for their effect upon macrophage complement receptor function. As shown in Fig. 7, bar C, supernates prepared in this fashion were inactive. To be certain that the Millipore filters had not simply absorbed the activity from these supernates, identical experiments were performed in which lymphocytes and macrophages were cocultivated with Millipore filters overlying the cells, and supernates generated in the standard fashion were preincubated with Millipore filters for 2 h at 37°C before transfer to assay macrophages. Supernates prepared and treated in these manners were as active as standard supernates (Fig. 7, bars D and E). Although it remains possible that effector macrophages may exert their effect upon lymphocytes by means of a very labile soluble mediator, these results strongly suggest that lymphocyte-macrophage contact is necessary for the generation of supernatant activity.

Is the Interaction between Lymphocytes and Macrophages Histocompatibility Restricted? Contact between lymphocytes and macrophages has been found to be necessary for thymocyte maturation (50, 57), for maximal T-cell proliferation in response to macrophage-bound antigen (48, 49, 53, 55), for the generation of carrier-specific helper T cells (45), for the production of lymphokines by T and B lymphocytes (33), and for IgG production by lymphocytes (42-44, 46). In most of these examples, macrophages and lymphocytes can cooperate only when they share certain histocompatibility specificities. In fact, in some experimental systems, efficient macrophage-lymphocyte contact does not occur unless the cells involved are identical at the I region of the H-2 complex. Because lymphocyte-macrophage contact appears to be required in our system, it was of interest to determine whether or not the collaboration of the two cell types was histocompatibility restricted.

Thymocytes and macrophages from BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were cocultivated for 24 h at 37°C in all possible combinations; some macrophages had previously ingested E(IgG). The activity of each culture supernate was assessed using assay macrophages from both BALB/c and C57BL/6 mice. As depicted in Table V, cultivation of allogeneic thymocytes with macrophages that had not phagocytized did not result in the elaboration of supernatant activity by thymocytes, indicating that, at least under the culture conditions employed, recognition of foreign histocompatibility antigens by lymphocytes was not a sufficient stimulus for the production and/or release of the active principle. Supernates from all cultures which contained thymocytes cocultivated with macrophages which had previously ingested E(IgG), however, were active regardless of the source of either cell type. These results indicate that there is no histocompatibility restriction involved in the cell-cell coop-

TABLE V
*Lack of Histocompatibility Restriction in the Generation of and Response to
 Active Supernates*

Effector macro- phages	H-2 Type		PI of E(IgM)C by assay macrophages
	Lymphocytes	Assay macro- phages	
d*	b	d	38
d*	b	b	41
b*	d	d	22
b*	d	b	41
d	d	d	335
d	d	b	205
d	b	d	350
d	b	b	399
b	d	d	413
b	b	d	308
b	d	b	371
b	b	b	265

* Effector macrophages were not previously incubated with E(IgG).

eration required for the generation of active supernates and that the response to supernatant activity is not dependent upon histocompatibility between the cells generating the activity and the cells responding to it.

Discussion

Peritoneal macrophages from mice that had been injected with thioglycollate medium were previously reported to ingest E(IgM)C (8). Because injection of thioglycollate medium into the mouse peritoneal cavity may have induced the influx of a population of mononuclear phagocytes with enhanced complement receptor activity, it could not be concluded from those studies that the complement receptor function of resident peritoneal macrophages had been, or could be, augmented. Results of the present experiments demonstrate that tissue macrophages can be induced to ingest particles via their complement receptors.

Induction of this property in macrophages was effected by a product(s) in supernates of cultures containing lymphocytes and specifically triggered macrophages. Several hours were required for both the generation and the expression of supernatant activity. Activity was generated when lymphocytes and macrophages interacted at physiologic temperature but not when the cells were cocultivated at 4°C. These initial results indicated that the activity assayed did not derive from nonspecific cellular metabolites or from alterations in trivial experimental conditions such as pH, tonicity, protein content, or ion concentration of the incubation media. Rather, they strongly suggested that either cellular metabolism, temperature-dependent cell-surface interactions, or both were required for the generation of the active product(s). Subsequent experiments revealed that both cellular metabolism and lymphocyte-macrophage surface interactions were required, for effector macrophages had first to phagocytize IgG-containing immune complexes and then to interact by a contact-dependent mechanism with T lymphocytes in order to signal the lymphocytes to elaborate the activity assayed.

Attachment and ingestion of particles by phagocytic cells are known to modulate several cellular functions, some of which are directly related to killing or degrading the ingested material. For example, phagocytizing macrophages synthesize increased quantities of lysosomal hydrolases (58); and neutrophils and macrophages are stimulated by the act of phagocytosis or by interaction of certain plasma membrane receptors with appropriate ligands to release lysosomal enzymes and to increase their rates of oxygen uptake and metabolism (41, 59). Certain other macrophage functions, which do not appear to be related to the disposal of ingested material, may also be augmented by phagocytosis. Phagocytosis is a potent inducer of monocyte synthesis and secretion of endogenous pyrogen (60). Gordon et al. (61) found that endotoxin-elicited mouse peritoneal macrophages were triggered to secrete plasminogen activator when the cells ingested either polystyrene latex beads or bacteria, and phagocytosis was subsequently found to trigger the synthesis and secretion of collagenase and elastase as well (62, 63). Unanue et al. (31) have shown that macrophages are induced by the act of phagocytosis to release a factor(s) that influences the behavior of both T and B lymphocytes. In contrast to our findings, however, induction of the macrophage property examined in each of these studies, although dependent upon phagocytosis, was independent of the means by which phagocytosis occurred.

That the metabolic events associated with phagocytosis of a particle by one type of plasma membrane receptor may be quite different from events associated with ingestion of a particle by a different means is suggested by recent work from two laboratories. Michl et al. (64) found that 2-deoxy-D-glucose blocked immunologically mediated phagocytosis by macrophages but had no effect upon the cells' ability to ingest particles via nonimmunologic means. Muschel et al. (65) found that the ingestion of IgG-coated E, but not of latex particles, could be modulated by treating macrophages with insulin or cyclic AMP. Because the physiology of phagocytosis varies depending upon the route by which a particle is phagocytized, it would not be surprising if different routes of ingestion were to regulate different macrophage functions. Yet there are only rare instances in which such functional selectivity is known to occur. *Rickettsia mooseri* (66), *Toxoplasma gondii* (67), and vaccinia virions (68) are readily ingested by macrophages, but only microorganisms that have been pretreated with immune serum are killed intracellularly. In these cases, lysosomal fusion with phagosomes appears to be dependent upon the interaction of particle-bound IgG with the macrophage, presumably via the cell's Fc receptors. Our results strengthen the hypothesis that metabolic events induced by phagocytosis of immunologically coated particles are different from those induced by phagocytosis of noncoated particles. They also demonstrate that metabolic events triggered by Fc receptor-mediated phagocytosis are different from those triggered by C3b receptor-mediated phagocytosis, for ingestion via the macrophages' complement receptors was not a sufficient stimulus for the effector macrophages' participation in the generation of supernatant activity.

The lymphocyte that participates in the generation of supernatant activity was shown to be the T lymphocyte. Both thymocytes and T-cell-enriched lymphocyte preparations were as effective as whole spleen cell populations, whereas B lymphocyte-enriched preparations were ineffective. In subsequent experiments, we found that the T lymphocyte was the cell which elaborated the active principle, for when thymocyte-macrophage cultures were split after 12 h of cocultivation and the cell types cultivated

separately, only thymocyte supernates were able to augment macrophage complement receptor function. In addition, treatment of thymocytes with concanavalin A resulted in the appearance in culture supernates of a similar activity (J. A. Griffin and F. M. Griffin, unpublished observation). These findings are consistent with the findings of many other investigators that most mediators that influence macrophage behavior are elaborated by T lymphocytes (16).

The results of experiments in which thymocytes were incubated in supernatant media from cultures containing Fc receptor-triggered macrophages and of experiments in which lymphocytes and macrophages were cocultivated in the same dish but were separated by a Millipore filter strongly suggest that lymphocyte-macrophage contact is required for macrophages to induce lymphocytes to elaborate the active material. Several investigators have previously shown that lymphocyte-macrophage contact is required for macrophages to exert certain effects upon T lymphocytes. Werdelin et al. (51), Nielson et al. (52), and Braendstrup et al. (53) have shown that intimate association occurs between sensitized T lymphocytes and macrophages in the presence of appropriate antigen and that physical contact is required for optimal lymphocyte blastogenesis to occur. Lipsky and Rosenthal (54-56) also demonstrated that macrophage-T lymphocyte contact is required for lymphocytes to respond to macrophage-bound antigen and further showed that the two cell types must be histocompatible. Van den Tweel and Walker (57) have recently shown that thymocytes undergo mitogen-induced blastogenesis only after being cocultivated with macrophages; macrophage-thymocyte contact appeared to be required but did not occur via histocompatibility antigens, because allogeneic, as well as autologous, macrophages were capable of imparting to thymocytes enhanced mitogen responsiveness. In our system, also, the requirement for lymphocyte-macrophage contact was independent of histocompatibility, for cells from mice with completely different H-2 specificities could cooperate to produce the activity. Therefore, although lymphocyte-macrophage contact is required for the generation of active supernates, the cellular interactions probably occur via surface moieties not encoded by the major histocompatibility complex.

Possible Physiologic Significance of Complement Receptor-Mediated Phagocytosis by Macrophages. It is becoming increasingly apparent that the cellular and humoral limbs of the immune system are not functionally separate entities. We have described in vitro conditions wherein these limbs may be tightly interwoven in a cyclic amplification of the function of each by the other. The phenomena described may occur in vivo and may have implications in the areas of inflammation, host defense against microbial pathogens, immune complex disease, and neoplasia.

The ability of macrophages to ingest via their complement receptors a particle coated with both IgG and C3b molecules may be advantageous, for in vivo phagocytic cells are bathed in IgG, which can adsorb to their Fc receptors and which, at concentrations less than those found in serum and tissue fluid, can impair the cells' ability to ingest IgG-coated particles in vitro (2, 5, 69). In addition, many microorganisms shed antigens which become bound by specific IgG; when these immune complexes are ingested by macrophages, the cells' ability to ingest the IgG-coated microbe by their Fc receptors may then be further impaired (70). The formation of immune complexes by shed microbial antigens and specific IgG may also diminish the quantity of antibody available to opsonize the microbe. Because most microor-

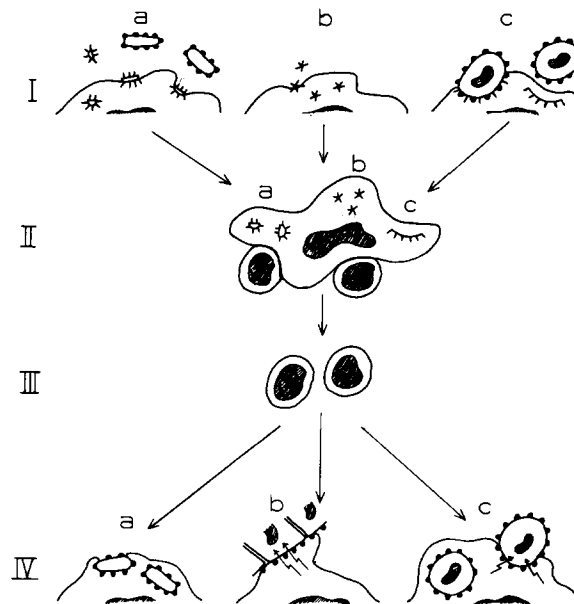


FIG. 8. Model for the possible significance of enhanced macrophage complement receptor function in: (a) host defense against microbial pathogens, (b) host cell injury in immune complex disease, and (c) destruction of neoplastic cells. Sequence (a): (step I) macrophages ingest shed bacterial antigen-IgG complexes (*), thereby (step II) acquiring the ability to interact with T lymphocytes such that (step III) T cells elaborate the lymphokine which (step IV) permits macrophages to ingest C3b (▲)-coated bacteria. Sequence (b): (step I) macrophages ingest soluble immune complexes (*) and thereby (step II) acquire the ability to interact with T lymphocytes such that (step III) T cells elaborate the lymphokine that (step IV) permits macrophages to damage host cells which are innocently coated with C3b at sites of inflammation. Sequence (c): neoantigens of malignant cells become bound by specific IgG (I), and C3b is deposited over the cells' surfaces. The neoplastic cells cap neoantigen-IgG complexes. Macrophages bind to neoplastic cells via the complexes and (step I) ingest the caps, thereby (step II) acquiring the ability to interact with T lymphocytes such that (step III) T cells elaborate the lymphokine which (step IV) permits macrophages to damage the C3b-coated neoplastic cells.

ganisms have surface moieties, e.g., polysaccharide or lipopolysaccharide, which fix complement via the alternative pathway, they become coated with C3b even when not coated with antibody (71). Macrophages, having ingested microbial antigen-IgG complexes, could trigger T lymphocytes to secrete the lymphokine that enhances macrophage complement receptor function. As a result, macrophages in the vicinity of these lymphocytes would acquire the ability to ingest the C3b-coated microorganisms (Fig. 8, sequence a).

A similar sequence of events may occur in immune complex diseases and result in host cell damage (Fig. 8, sequence b). Macrophages that ingest immune complexes may interact with T lymphocytes, resulting in the elaboration of a product that alters the macrophages' interaction with C3b-coated cells. Host cells which have become innocently coated with C3b at sites of immune complex-induced inflammation may then be attacked by these macrophages, either by phagocytosis or by enhanced release of lysosomal enzymes, superoxide anion, or hydrogen peroxide. Rapid reversal of the effect upon macrophage complement receptor function after dissipation of the active product, which would occur when inflammation had been sufficiently cleared, would serve as a control mechanism to prevent continued destruction of host tissues.

Finally, our findings may be pertinent to tumor cell destruction by macrophages (Fig. 8, sequence c). Many neoplastic cells express on their surfaces antigens not present on normal cells (72, 73). Host antibody to these antigens is elicited and binds specifically to these neoantigens; in addition, complement components, including C3b, are subsequently deposited over the cells' surfaces. Some neoplastic cells are capable of capping cell surface immune complexes (74). We have shown (39) that macrophages can phagocytize capped immune complexes from the surfaces of some cells without harming the cells. In the case of a neoplastic cell, such a series of events would result in a cell diffusely coated with C3b but with neither antibody nor abnormal determinants on its surface. Normal macrophages would be able to bind to the C3b-coated cell, but would not ingest it, and would probably release the cell when C3b inactivator cleaved C3b to C3c and C3d. Schreiber and Frank (75) have shown that the interaction of normal macrophages with C3b-coated E does not result in E damage; it is likely that a similar interaction of normal macrophages with C3b-coated tumor cells would likewise not harm the cells. However, the ingestion by macrophages of immune complexes from the surface of a tumor cell may permit the macrophages to trigger T lymphocytes to elaborate a product that permits these or other macrophages to interact more aggressively with C3b-coated cells. These macrophages may then be able to destroy C3b-coated tumor cells, either by phagocytosis and intracellular killing or by extracellular cytotoxic mechanisms.

Summary

The function of complement receptors of mouse peritoneal macrophages was converted in vitro from mediating only attachment, to promoting both attachment and ingestion of C3b-coated particles. Enhancement of macrophage complement receptor function was achieved by treating freshly explanted macrophages with supernates from cultures containing T lymphocytes and appropriately triggered macrophages. Fc receptor-mediated phagocytosis by macrophages was required for the production of active supernates, for neither ingestion via the cells' complement receptors nor ingestion via nonimmunologic means was a sufficient stimulus for the macrophages' participation in the generation of supernatant activity. Fc receptor-triggered macrophages interacted by a contact dependent, but histocompatibility independent, mechanism with T lymphocytes, thereby signalling the lymphocytes to elaborate the active product. The possible significance of enhanced macrophage complement receptor function in inflammation, host defense against microbial pathogens, immune complex disease, and neoplasia is discussed.

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